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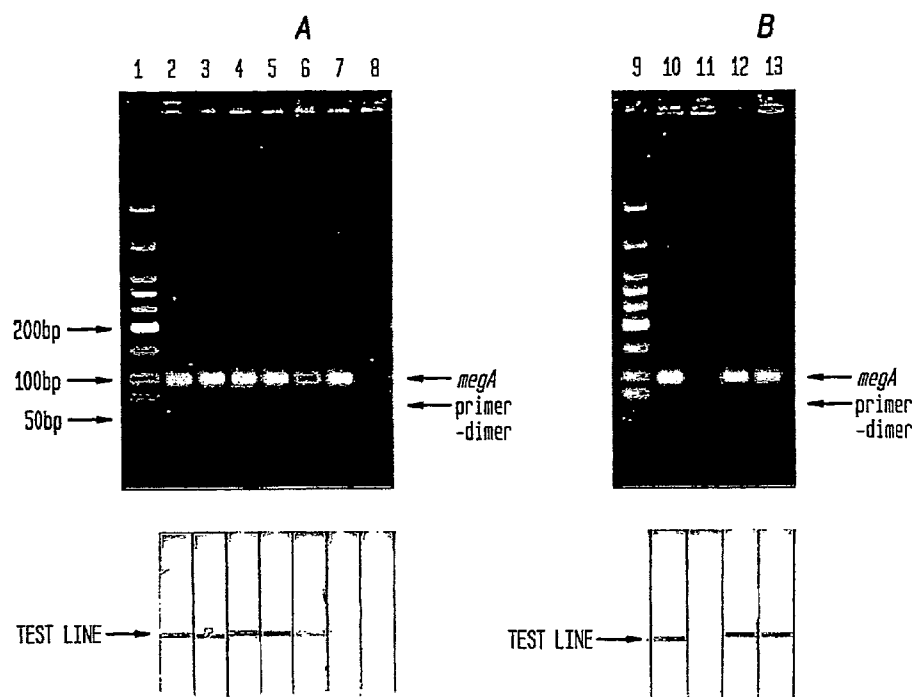
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(54) Title: A METHOD OF ASYMMETRIC HELICASE-DEPENDENT AMPLIFICATION FOR PROBE-BASED DETECTION OF TARGETS



(57) Abstract: A method is provided for performing asymmetric helicase-dependent (HDA) amplification and detecting pathogens using asymmetric HDA and nucleic acid probes.

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A Method of Asymmetric Helicase-Dependent Amplification for Probe-Based Detection of Targets

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BACKGROUND

Molecular diagnostics frequently utilize sequence-specific primers for the amplification and detection of a target nucleic acid. Unfortunately, the formation of primer-dimers can reduce the yield of amplicons in an amplification reaction and can generate false positive results in a diagnostic test. The sensitivity of a diagnostic test is also affected by primer-dimer formation where the diagnostic test relies on the binding of single-stranded probes to single-stranded amplicons. Primer-dimer formation inhibits this reaction and can give rise to false negative results.

Polymerase chain reaction (PCR) amplification (for example, see U.S. Pat. Nos. 4,683,195, 4,683,202 and 4,800,159) involves the repeated, complete denaturation of double-stranded DNA by heat during the amplification process. The repeated cycles of heat denaturation required for amplification also permits the binding of a labeled probe to the amplicon generated. This avoids the problems associated with false positive results generated by primer-dimers and also enhances the specificity of nucleic acid analysis.

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Helicase-dependent amplification (HDA) has advantages over polymerase chain amplification that include the removal of dependence on thermocycling for amplification (see, for example, U.S. patent publication 2004/0058378, Vincent et al., *EMBO Reports* (2004) and An et al., *J. Biol. Chem.* 280: 28952-28958 (2005)). Amplification of target polynucleotides without requiring

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thermocycling is an attractive feature for diagnostic tests where testing facilities may not have access to a thermocycler. However, the use of HDA for diagnostic tests is adversely affected by primer-dimer formation, which reduces the amount of single-strand
5 amplicon available for hybridizing to a probe. This in turn adversely affects the sensitivity of the diagnostic test.

SUMMARY

10 In an embodiment of the invention, a method is provided for detecting a target polynucleotide segment in a sample. Applications of this method include diagnosis of a pathogen, detecting a mutation in a genomic DNA such as human genomic DNA and single polynucleotide detection. The method includes (a) obtaining a
15 single-stranded amplicon using HDA and a first primer at a concentration of at least three times greater than the concentration of a second primer wherein the first and second primers hybridize to opposite strands at opposite ends of the target polynucleotide segment; and (b) hybridizing a single-strand probe to the single-
20 stranded amplicon so as to detect the target polynucleotide segment.

In an embodiment of the invention, the amplification is real-time amplification and the probe is a labeled probe. An example of a
25 labeled probe is a probe with a fluorescent label. Alternatively, amplification may be detected by end-point analysis using either labeled or unlabelled probes. End-point analysis can be achieved by a lateral-flow device.

30 HDA can be conducted under isothermal conditions for example in the range of 20°C and 75°C or within a range of 60°C and 66°C. The second primer may be provided at concentrations

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within a range of 25 nM -75nM. Tte UvrD helicase may be used in the HDA reaction.

In an embodiment of the invention, a composition is provided that is characterized by a first primer and a second primer, wherein the ratio of the first primer is at least 3:1 with respect to the second primer. The second primer may be present in the reaction at a concentration in the range of 25 nM -75nM. The first and second primers are capable of hybridizing to opposite strands of a polynucleotide sequence at opposite ends of a target polynucleotide segment. The composition also contains a helicase such as Tte UVRD helicase in an effective concentration for amplifying a polynucleotide to produce a single-strand amplicon.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-1B show a comparison of RT fluorescent-based detection of Herpes Simplex Virus (HSV) using asymmetric versus symmetric HDA.

Figure 1A and B shows melt curve plots resulting from real-time amplification where cycle number is varied in Figure 1A and temperature is varied in Figure 1B. Real-time symmetric and asymmetric HDA were carried out on an ABI 7300 system (Applied Biosystems (ABI, Foster City, CA) with a pair of glycoprotein B gene specific primers and 5×10^4 copies of pHSV1GB template DNA containing the cloned HSV-1 glycoprotein B gene fragment. A primer ratio of 1:1 was used in the symmetric amplification reactions. A primer ratio of 4:1 (excess primer to limiting) was used in the asymmetric amplification reactions. The concentrations of HSV-1 probe tested in the HDA reactions are:

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25 nM for reactions samples 1 & 5;

50 nM for samples 2 & 6;

100 nM for samples 3 & 7;

200 nM for samples 4 & 8.

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Real-time detection was performed with a fluorescent-labeled HSV-1 MGB probe, evaluated at different concentrations, and followed by melt-curve analysis.

Samples 1–4 show the results of symmetric amplification reactions;

10 Samples 5 –8 show the results of asymmetric amplification reactions.

Figure 2 shows real-time fluorescent probe based detection of HSV strains using asymmetric HDA. Real-time asymmetric HDA was
15 analyzed using an ABI 7300 system with a pair of primers that target the glycoprotein B gene of HSV.

Figure 2A shows a real-time amplification plot. Figure 2B shows a standard curve plot of amplified HSV-2. Serial dilutions of the pHSV2GB plasmid containing the cloned glycoprotein B gene
20 fragment of HSV-2 were used as the template for standard curve analysis. Real-time detection was performed with a HSV-2 fluorescent-labeled MGB probe.

Figure 2C and 2D shows a real-time amplification for HSV stain-typing. An HSV-1 MGB fluorescent-labeled probe that contains
25 a single base pair mismatch with the corresponding HSV-2 sequence was used in the melt curve analysis for HSV typing. Asymmetric HDA was carried out with either 5×10^2 copies of pHSV1GB target.

30 Figures 3A and 3B show the results of an assay to detect methicillin-resistant *staphylococcus aureus* (MRSA) by asymmetric

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HDA on a lateral flow strip. Asymmetric HDA was carried out using a pair of *mecA* gene specific primers in the presence of a biotin-labeled probe in a total volume of 50 μ l. After HDA, 10 μ l of the products were separated on a 2% agarose gel (Figure 3A). Another 10 μ l of the products were incubated with a gold conjugated anti-biotin antibody and then run on a DNA test strip pre-stripped with an anti-DNP antibody (Figure 3B).

Figure 3A shows the limit of detection (LOD) of the HDA-based lateral-flow MRSA assay.

10 Lanes 2 – 8 show HDA products generated from a serial dilution of genomic DNA purified from the MRSA strain, Mu50.

Lane 2: 5×10^5 copies;

Lane 3: 5×10^4 copies;

Lane 4: 5×10^3 copies;

15 Lane 5: 5×10^2 copies;

Lane 6: 5 x 10 copies;

Lane 7: 5 copies;

Lane 8: 0 copies.

Figure 3B shows the specificity of the HDA-based lateral-flow MRSA assay performed in the presence of non-MRSA *S. aureus* (SA) cells.

Lane 10: 10^6 cells from the MRSA strain, MW2.

Lane 11: 10^6 *Staphylococcus aureus* (SA) cells as the negative control.

25 Lane 12: 10^2 of MRSA cells.

Lane 13: 10^2 MRSA cells plus 10^6 SA cells.

Lanes 1, 9: 250 ng of Low Molecular Weight DNA Ladder (NEB, Ipswich, MA).

DETAILED DESCRIPTION OF THE EMBODIMENTS

In order to overcome the limitations of primer-dimer formation and inefficient probe-binding to an amplicon, we have developed an asymmetric amplification method that utilizes HDA, a technique that has been described in detail in US appln No 11/327,175. This method involves the use of a range of optimized concentrations of the forward and reverse primers to increase the percentage of single-stranded amplicons from target polynucleotides to facilitate the binding of a single-stranded labeled or unlabeled sequence-specific probe to the amplicon. The use of at least 3 fold excess of one primer over another results in the preferential amplification of one of the target strands, creating an excess of single-stranded DNA. The increased quantity of single-stranded amplicon produced by HDA allows for the more efficient binding of single-stranded amplicon to single-stranded DNA probes for detection of targeted sequences. This increases the specificity and utility of the probe-based detection methods using HDA by eliminating false-positives that result from non-specific amplification and primer-dimer formation. The combination of the asymmetric HDA method with probe-based detection methods increases the utility of HDA for molecular diagnostics, forensics, and environmental testing.

Asymmetric HDA can be performed in conjunction with a variety of labeled or unlabeled sequence-specific probes, well known in the art, for the purpose of detecting the target nucleic acid product generated from the amplification reaction. Sequence-specific detection of the product is preferred, as this ensures that the product generated from the amplification reaction is the

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intended product rather than one resulting from spurious non-specific amplification.

The experiments described herein were performed using a
5 real-time thermocycler, the ABI 7300™ (ABI, Foster City, CA),
because an isothermal real-time fluorescent detector was not
available. This detector requires at least some degree of
thermocycling. Because HDA does not normally utilize
thermocycling, the temperature alternated between 65°C and 66°C,
10 corresponding to the minimum temperature difference necessary to
utilize the detection function capabilities of the detector. The
amplification reactions could be performed in any device capable of
measuring the signal generated from the specific type of labeled or
unlabelled probe.

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The labeled probe can be a fluorescent probe comprised of
DNA, RNA or a hybrid of DNA/RNA, for example, an MGB Eclipse
probe (Nanogen, San Diego, CA), a HyBeacon probe (LGC, London,
UK), a Hybridization probe (Roche, Basel, Switzerland), a Molecular
20 Beacon probe (Tyagi et al. *Nature Biotechnology* 14:303-8 (1996)),
or a TaqMan probe (ABI, Foster City, CA). These probes may
contain a fluorophore on either the 5'- or 3'-end or a fluorophore on
either end in combination with a quencher on the opposite end.
These probes may contain a fluorophore on an internal nucleotide or
25 may be labeled with multiple fluorophores throughout the sequence.
These probes may also contain other moieties such as a biotin,
streptavidin, DNP (dinitrophenyl) or any other group capable of
being conjugated to DNA or RNA and useful for detection. The
probes may be comprised of standard or modified nucleic acids or
30 analogs thereof (e.g. peptide nucleic acids (PNAs) (Nielsen, *Mol.*
Biotechnol 26:233-48 (2004)) or locked nucleic acids (LNAs)

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(Petersen et al. *Trends Biotechnology* 21:74-81 (2003)). The labeled probes may be used in real-time detection of product formation, end-point analysis of product formation or with lateral-flow-based detection methods.

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Examples of unlabeled probes include unmodified or modified DNA, RNA, or combinations of DNA and RNA or analogs thereof including peptide nucleic acids (PNAs) or locked nucleic acids (LNAs)). Probes may be linear, hairpin sequences, or other conformational alterations (e.g. a Molecular Beacon structure with no label).

10

Detection using an unlabeled probe can be achieved by high-resolution melt-curve analysis, physical separation based upon size differences (e.g. gel electrophoresis) or other methods known in the art. In addition, combinations of labeled and unlabeled probes may be used in order to simultaneously differentiate between targets that contain mutations or multiple, unique strains of pathogens.

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Asymmetric Helicase-Dependent Amplification

Asymmetric HDA uses unequal concentrations of primers to preferentially generate single-stranded amplicon for hybridization to a probe for detection of a target polynucleotide segment. (A polynucleotide segment here refers to a segment, which could be a whole or part of a polynucleotide molecule. The polynucleotide segment may include a DNA/RNA or DNA/RNA hybrid, or modified DNA, RNA or DNA/RNA hybrid molecule) Asymmetric HDA may be performed isothermally at a temperature in the range of 20°C – 75°C, more specifically in a range between 60°C – 66°C for a

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thermophilic HDA reaction (An et al., *J. Biol. Chem.* 280:28952-28958 (2005)). The forward primer is provided at a higher concentration than the reverse primer, which serves as the limiting primer. Alternatively, the reverse primer can be provided in at least
5 three fold excess where the forward primer serves as the limiting primer. Depletion of the limiting primer during exponential amplification results in linear synthesis of the product generated from the excess primer. This leads to accumulation of the single-stranded amplicon that originated from the excess primer. The
10 limiting primer is provided in the range of 25 nM – 75 nM (for example, 50 nM). The excess primer is provided at a concentration that is in the range of approximately 1.5 to 10 times that of the limiting primer (for example, 200 nM). In one embodiment, the excess primer is provided at a concentration that is four times
15 higher than the concentration of the limiting primer.

In another embodiment of the invention, asymmetric HDA is performed in the presence of a sequence-specific DNA probe to detect or quantify nucleic acid targets. One example of this is the
20 combination of asymmetric HDA with an MGB Eclipse fluorescent probe specific for the target sequence for real-time quantitative analysis. Quantitative analysis is performed by determining the amount of fluorescence present in a sample after the subtraction of background fluorescence from each reaction.

25

The described method of asymmetric HDA performed in conjunction with sequence-specific probe detection is particularly useful for applications (e.g., strain typing or identification) in which the target nucleic acid is to be discriminated from one or more
30 similar variants (e.g., a polynucleotide with a single nucleotide substitution, a human genetic variant, human gene mutation or

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multiple infectious agents). For example, asymmetric HDA combined with an MGB Eclipse probe can be used for discrimination of a single nucleotide polymorphism. In such applications, MGB probes are selected so that the melting temperature (T_m) of the

5 MGB probe-target hybrid that contains a perfect match to the target sequence is higher (e.g. 67°C) than the asymmetric HDA reaction temperature (e.g. 65°C). The T_m of the hybrid formed between the MGB probe and the target that contains one mismatch is lower (e.g. 55°C) than the temperature of the asymmetric HDA reaction

10 (65°C). As a result, the MGB probe will only hybridize with the perfectly matched amplicon at the reaction temperature of 65°C, resulting in a fluorescent signal that can be detected in a real-time HDA assay performed in a machine capable of measuring fluorescence. In contrast, the probe does not bind to an amplicon

15 that has a single mismatched base pair at the reaction temperature of 65°C and therefore no fluorescent signal is detected in a real-time HDA assay.

The results obtained by this method can be verified by

20 performing a melt-curve analysis following the asymmetric HDA reaction, in which the melting temperature of MGB probe-target duplex is determined (see Figures 1A and 1B). As a result, the T_m peak of the MGB probe-target hybrid formed from the perfect match (e.g. 67°C) is differentiated from the T_m peak of the MGB probe-

25 target hybrid that contains a mismatch (e.g. 55°C).

The sequence-specific probe can also be labeled with a small molecule and used in end-point analysis to determine the presence or absence of a target. In one embodiment, the sequence-specific

30 probe is labeled with biotin and an excess of one primer labeled with DNP is provided. Asymmetric HDA is performed and the

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products generated are used for lateral-flow detection. Using the lateral flow detection method, the amplified product is incubated with a gold-conjugated antibody to the biotin-labeled probe. After incubation, the sample is applied to a lateral-flow test strip that is pre-stripped with an antibody against the DNP-labeled primer. A positive result is detected as a visible line on the test strip only when the probe is hybridized to the single-stranded amplicon generated by the excess primer during asymmetric amplification. This ensures that detection is specific for the amplified product.

The following examples are provided to experimentally illustrate certain embodiments of the invention, but are not intended to limit the invention.

All references cited herein, as well as U.S. provisional application number 60/791,987 filed April 14, 2006, are incorporated by reference.

EXAMPLES

Example I: Demonstration of the Requirement of Asymmetric Primer Concentrations for Efficient Probe-Based Detection with the HDA method

HDA was carried out using a pair of primers that target the sequence of the glycoprotein B gene of Herpes simplex virus (HSV). Symmetric HDA reactions were performed with equal amounts of forward and reverse primers and compared with reactions performed with unequal amounts of forward and reverse primers (asymmetric HDA). The symmetric HDA reactions used 75nM of both the forward and reverse primers. In asymmetric HDA reaction,

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the excess primer was provided at a concentration of 200 nM, which is 4-times higher than that the concentration of the limiting primer (50 nM). Plasmid pHSV1GB was used as the template for the HDA assay. pHSV1GB was constructed by first amplifying HSV-1 clinical specimens obtained from Lahey Clinic (Burlington, MA) with HDA using GBF1: TTCAAGGAGAACATCGCCCCGTACAA (SEQ ID: 1) and GBR1: TAAACTGGGAGTAGCGGTGGCCGAAC (SEQ ID: 2) as the primers. The amplified HDA products were then cloned into the pCRII-TOPO vector (Invitrogen, Calsbad, CA). The HSV-1 minor groove binder (HSV-1 MGB) probe was synthesized by Nanogen, Inc., San Diego, CA. and designed to target the sequence between the two amplification primers. The probe was labeled with 6-carboxy-fluorescein (FAM) and MGB at the 5'-end, and Eclipse Dark Quencher at the 3'-end. The amount of HSV-1 MGB probe was titrated from 25 nM to 200 nM into the real-time HDA assay.

The real-time HDA reaction with a total volume of 50 µl was set up by first making two reaction mixtures: Mix A and Mix B.

Mix A was prepared in a sterile optical tube (ABI, Foster City, CA) by combining:

- 2.5 µl 10X HDA buffer (see below*)
- 5 µl pHSV1GB (10^4 copies /µl)
- 0.75 µl 5 µM GBF1 primer:
 - TCAAGGAGAACATCGCCCCGTACAA (SEQ ID NO:1) (for symmetric HDA) or 0.5 µl 5 µM GBF1 primer:
 - TCAAGGAGAACATCGCCCCGTACAA (SEQ ID NO:1) (for asymmetric HDA)
- 0.75 µl 5 µM GBR1 primer:
 - TAAACTGGGAGTAGCGGTGGCCGAAC (SEQ ID NO:2)

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(for symmetric HDA) or 2 μ l 5 μ M GBR1 primer:

TAAACTGGGAGTAGCGGTGGCCGAAC (SEQ ID NO:2)

(for asymmetric HDA)

2.5 μ l 0.5 - 4 μ M HSV-1 MGB probe:

5 MGB/FAM-ACTACAAAGACGTCACCG-Q (SEQ ID NO:3)

13.5 μ l dH₂O (for symmetric HDA) or 12.5 μ l dH₂O (for asymmetric HDA)

Total volume: 25 μ l

10 Mix B was prepared in another sterile microtube by combining:

2.5 μ l 10X HDA buffer (see below*)

2 μ l 100 mM MgSO₄

4 μ l 500 mM NaCl

15 2 μ l 10 mM dNTP

1.5 μ l 100 mM dATP

1 μ l ROX dye (50X, Invitrogen, Carlsbad, CA)

2.5 μ l *Bst* DNA Polymerase, LF (8 units/ μ l, NEB, Ipswich, MA)

1 μ l Tte-UvrD helicase (150 ng/ μ l) (Tang et al. *J. Biol. Chem.*

20 2005, 280:28952-8)

8.5 μ l dH₂O

Total volume: 25 μ l

*10X HDA buffer contains 100 mM KCl, 200 mM Tris-HCl (pH 8.8 at 25°C).

25

Mix A was first denatured at 95 °C for 2 min. After cooling Mix A on ice, Mix B was added into Mix A. The real-time asymmetric HDA reaction was carried out on an ABI 7300™ real-time machine (ABI, CA) with the following settings:

30 reporter dye: FAM;

quencher: none;

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passive reference dye: ROX.

Because the ABI machine is a thermocycler, modifications to the program was made to perform an HDA reaction. The instrument
5 was programmed for 60 "cycles", and each cycle consisted of two segments: a 66°C incubation for 5 seconds followed by a 65°C incubation for 1 minute and 55 seconds. Data collection and real-time analysis were carried out during the 65°C step. This was followed by using the default dissociation stage setting for melt-
10 curve analysis with a starting temperature of 50°C.

Results are illustrated in Figure 1. When the probes were used in the regular, symmetric HDA reactions (samples 1 – 4), detection signal increased along with an increase in probe concentration as
15 shown in the real-time graph (see Fig. 1 panel A, samples 1, 2, 3, 4). In comparison, asymmetric HDA generated significantly higher fluorescent signals than the regular HDA assay. The fluorescent signal generated from asymmetric HDA reaction (samples: 5 – 8) is 8.0, 7.3, 6.6, and 5.4 times that generated from the symmetric
20 reactions (samples: 1 – 4) using an MGB probe concentration of 25 nM, 50 nM, 100 nM, and 200 nM respectively (Fig. 1A). Enhanced fluorescence from the asymmetric reactions is also seen in the melt-curve analysis plot (Fig. 1B). Moreover, the C_t of the symmetric HDA reactions are increased compared to those
25 generated from the asymmetric HDA reactions for the same probe concentration. The C_t is a measure of the number of "cycles", which for HDA, corresponds to the amount of time required to generate enough fluorescent signal to cross the threshold and be measurable above background. An increase in C_t value corresponds to a
30 decrease in the fluorescence generated from the reaction. Thus, it was found that asymmetric HDA coupled with an HSV MGB probe

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greatly improved the quality in addition to enhancing the amount of the fluorescent signal for real-time detection of HSV. It was also noted that the HSV-1 MGB probe with a concentration higher than 100 nM (e.g. 200 nM) resulted in an inhibitory effect on the HDA reaction generating a delay in the real-time amplification plot (Fig. 1).

Example II: Use of fluorescent probe-based asymmetric HDA for real-time, quantitative detection and genotyping of Herpes Simplex Virus (HSV)

In this example, asymmetric HDA was carried out with a pair of GBF/GBR primers (SEQ ID. Nos. 1 and 2) that targeted a portion of the sequence of the glycoprotein B gene that is conserved between the HSV-1 strain and the HSV-2 strain. In the asymmetric reaction, the ratio of excess primer to the limiting primer is 4:1. The intra-primer region of the amplicon contains three mismatches between HSV-1 and HSV-2. Two MGB Eclipse probes (Nanogen, Inc) were designed based on this intra-primer region to discriminate between the sequences:

HSV-1: GTTCAAGGCCACCATGTACTACAAAGACGTC
ACCGTTTTCGCAGGTGTG: (SEQ ID. No. 8),
HSV-2: ATTCAAGGCCACCATGTACTACAAAGACGTG
ACCGTGTCGCAGGTGTG : SEQ ID. No 9)

Each of the probes was labeled with both FAM and the MGB at the 5'-end and the Eclipse Dark Quencher at the 3'-end. The HSV-1 MGB probe (SEQ ID NO:3) matches perfectly with the HSV-1 sequence but contains a single mismatch with the corresponding HSV-2 sequence, while the HSV-2 MGB probe is perfectly matched

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with the HSV-2 sequence but contains a single mismatch with the corresponding HSV-1 sequence. The HDA target the following sequences:

5 HSV-1: TTCAAGGAGAACATCGCCCCGTACAAG
TTCAAGGCCACCATGTACTACAAAGACGTCACCGTTTCGCAGGTGTG
GTTTCGGCCACCGCTACTCCCAGTTTA (SEQ ID NO:10),
HSV-2: TTCAAGGAGAACATCGCCCCGTACAAA
TTCAAGGCCACCATGTACTACAAAGACGTG ACCGTGTCGCAGGTGTG
10 GTTCGGCCACCGCTACTCCCAGTTTA (SEQ ID. No:11).

These sequences from HSV-1 and HSV-2 were generated by amplification from clinical specimens obtained from the Lahey Clinic (Burlington, MA). The sequences were amplified using the pair of
15 forward and reverse primers developed for the HDA assay (SEQ ID: 1 and SEQ ID: 2) and cloned into the pCRII-TOPO vector (Invitrogen, Calsbad, CA). The sequences were verified by DNA sequencing. The two plasmids generated were named pHSV1GB for the cloned glycoprotein B amplicon from the HSV-1 strain, and
20 pHSV2GB, for the cloned glycoprotein B amplicon from the HSV-2 strain. The plasmids were used as templates for the real-time asymmetric HDA assays. For standard curve analysis, serial dilutions of pHSV2GB from 5×10^6 copies down to 50 copies were used as templates, and the HSV-2 MGB probe was used as the
25 detection probe. Real-time fluorescent signal was detected when the HSV-2 MGB probe specifically bound to the single-stranded DNA generated during asymmetric HDA with the HSV-2 template.

For HSV typing, samples containing either 500 copies of
30 pHSV1GB or pHSV2GB, or 500 copies of both were used as template and the HSV-1 MGB probe was selected as the detection

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probe. Discrimination between the HSV-1 plasmid and the HSV-2 plasmid after asymmetric HDA was determined by melt curve analysis of the products generated. The HSV-2 single-stranded amplicon dissociates from the MGB probe at a lower temperature
5 than that for the HSV-1 due to the one mismatch between the HSV-1 probe and the corresponding HSV-2 sequence.

The real-time asymmetric HDA reaction with a total volume of 50 μ l was set up by assembling two master mixes: Mix A and Mix B.
10 Mix A was prepared in one sterile optical tube (ABI) by combining:
2.5 μ l 10X HDA buffer (see below*)
5 μ l HSV template
0.5 μ l 5 μ M GBF1 primer (SEQ ID NO:1)
2 μ l 5 μ M GBR1 primer (SEQ ID NO:2)
15 1 μ l 4 μ M HSV-2 MGB probe (SEQ ID NO:4, for standard curve analysis) or 1 μ l 4 μ M HSV-1 MGB probe (SEQ ID NO:3, for HSV typing)
14 μ l dH₂O
Total volume: 25 μ l
20 For the negative control reaction, replace HSV template with 5 μ l dH₂O.

Mix B was prepared in another sterile microtube by combining:
25 2.5 μ l 10X HDA buffer (see below*)
2 μ l 100 mM MgSO₄
4 μ l 500 mM NaCl
2 μ l 10 mM dNTP
1.5 μ l 100 mM dATP
30 1 μ l ROX dye (50X, Invitrogen)
2.5 μ l Bst DNA Polymerase, LF (8 units/ μ l, NEB, Ipswich, MA)

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1 μ l Tte-UvrD helicase (150 ng/ μ l)

8.5 μ l dH₂O

Total volume: 25 μ l

*10X HDA buffer contains 100 mM KCl, 200 mM Tris-HCl (pH

5 8.8 at 25°C).

Mix A was denatured at 95 °C for 2 min. After cooling Mix A on ice, Mix B was added to Mix A. The real-time asymmetric HDA reaction was carried out in an ABI 7300™ real-time machine with
10 the following settings:

reporter dye: FAM;

quencher: none;

passive reference dye: ROX.

15 Because the ABI machine is a thermocycler, modifications to the program have to be made to perform an HDA reaction. The instrument was programmed for 60 "cycles", and each cycle consisted of two segments: a 66°C incubation for 5 seconds followed by 65°C incubation for 1 minute and 55 seconds. Data
20 collection and real-time analysis was carried out during the 65°C step. This was followed by using the default dissociation stage setting for melt curve analysis with a starting temperature of 35°C.

25 The results obtained are illustrated in Fig. 2. Amplification was observed in real-time from samples containing HSV-2 template from 5×10^6 copies down to 50 copies but not from the non-template control (NTC) samples (Fig. 2A). The standard curve plot generated gave a significant correlation coefficient factor ($R^2 = 0.999$) (Fig.
30 2B). This demonstrates the ability of asymmetric HDA to be used in

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conjunction with a fluorescent MGB probe for use in quantitative real-time detection of HSV.

The HSV-1 MGB probe was utilized for genotyping analysis, rather than the HSV-2 MGB probe. Fluorescent signal during amplification was detected from samples containing the HSV-1 template, both the HSV-1 and HSV-2 templates, but not from the sample that contained only the HSV-2 template or the negative control (NTC) that did not contain any template (Fig. 2C). After amplification, melt curve analysis of the products generated showed a peak corresponding to a T_m of 67 °C in the sample that contained either the HSV-1 template or mixed HSV-1 plus HSV-2 template. The peak with a T_m of 67°C indicates the presence of the target amplicon as detected by the hybridization of the HSV-1 MGB probe. Melt-curve analysis also indicated the presence of a peak with a T_m of 55°C in samples that contained the HSV-2 target, corresponding to the less efficient binding of the HSV-1 MGB probe to the HSV-2 amplicons, which contain a single mismatch. (Fig. 2D). This result demonstrates the ability to utilize asymmetric HDA in conjunction with fluorescent MGB probes followed by melt-curve analysis to perform strain-typing of the two HSV viruses for diagnostic purposes. .

Example III: Use of a sequence-specific probe and asymmetric HDA to detect MRSA on lateral flow strips

In this example, the HDA assay was carried out with a pair of primers.

LSAMF2 primer (SEQ ID NO:5)

30 DNP- GAAAAATGATTATGGCTCAGGTACTGC
and SAMR2 primer (SEQ ID NO:6)

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TGGATAGACGTCATATGAAGGTGTGCT

These primers targeted the *mecA* gene in *S. aureus* (SA) (Chambers et al. *Clin. Microbiol. Rev.* 1997: 10: 781-91). The *mecA* gene is an indicator of methicillin resistance. Hospital-acquired MRSA is a significant and increasing problem and methods to specifically and rapidly detect this deadly bacteria are greatly needed. The asymmetric HDA assay performed to detect the *mecA* gene used an "excess primer" that was provided at a concentration 4 times the concentration used for the limiting primer. The excess primer was labeled with a 2, 4-dinitrophenyl (DNP) group on the 5'-end ((New England Biolabs, Inc. (NEB), Ipswich, MA)).

The SAM2P1 probe developed to target the *mecA* sequence was labeled with a biotin on the 3'-end (NEB, Ipswich, MA). SAM2P1 probe GTGCTAATAATTACCTGTTTGAGGGT-Biotin (SEQ ID NO:7)

The sequence of the detection probe is complementary to the intra-primer region of the product generated from the excess primer. To determine the limit of detection (LOD) of the HDA-based MRSA assay, serial dilutions of purified genomic DNA from the MRSA strain Mu50 (ATCC 700699D) from 5×10^5 copies down to 5 copies were used as template. To evaluate the specificity of the HDA-based MRSA assay, an HDA reaction was performed in the presence of non-resistant SA cells (*Staphylococcus aureus* strain ISP 794 from Massachusetts General Hospital, Boston, MA) that lacked the *mecA* gene. The asymmetric HDA product was subsequently applied to a lateral-flow test strip (DNA test strip from British BioCell International, UK) for detection.

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The asymmetric HDA reaction with a total volume of 50 μ l was prepared by making two master mixes: Mix A and Mix B. Mix A was prepared in one sterile microcentrifuge tube by combining:

- 2.5 μ l 10X HDA buffer (see below*)
 - 5 μ l MRSA or SA template
 - 2 μ l 5 μ M LSAMF2 primer (SEQ ID NO:5)
 - 0.5 μ l 5 μ M SAMR2 primer (SEQ ID NO:6)
 - 1 μ l 1 μ M SAM2P1 probe (SEQ ID NO:7)
 - 14 μ l distilled H₂O
 - 10 Total volume: 25 μ l
- For the negative control reaction, replace MRSA template with 5 μ l dH₂O.

- Mix B was prepared in another sterile microtube by
- 15 combining:
 - 2.5 μ l 10X HDA buffer (see below*)
 - 2 μ l 100 mM MgSO₄
 - 4 μ l 500 mM NaCl
 - 2 μ l 10 mM dNTP
 - 20 1.5 μ l 100 mM dATP
 - 2.5 μ l Bst DNA Polymerase, LF (8 units/ μ l, NEB, Ipswich, MA)
 - 1 μ l Tte-UvrD helicase (150 ng/ μ l)
 - 9.5 μ l dH₂O
 - Total volume: 25 μ l
- 25 *10X HDA buffer contains 100 mM KCl, 200 mM Tris-HCl (pH 8.8 at 25°C).

- Mix A was denatured at 95 °C for 2 min. After cooling Mix A on ice, Mix B was added to Mix A. The asymmetric HDA reactions
- 30 were carried out at 65 °C for 120 min. 10 μ l of the amplification products were analyzed on a 2% agarose gel. Another 10 μ l of the

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amplification products were transferred into the microwells of a 96-well microtitre plate for the lateral-flow assay (Fig. 1, lower panel). Immunogold conjugate monoclonal anti-biotin antibody (10 OD/ml, British Biocell International (BBI)) was first diluted in phosphate buffered saline (PBS, pH 7.2, 1% w/v BSA, 0.5%w/v Tween 20) to a concentration of 0.7 OD/ml. 50 μ l of the diluted conjugate was then mixed with the 10 μ l aliquot of the amplification products in the microwells and incubated at room temperature for 5 minutes. Half dipsticks (lateral-flow strips that do not contain an application pad) (British BioCell International, UK) that were pre-stripped with the anti-DNP monoclonal antibody British BioCell International, UK) were then placed in the microwells containing the amplification products and left to develop at room temperature for 30 minutes.

The results obtained are illustrated in Fig. 3. A DNA fragment of approximately 100 bp was observed on the agarose gel in agreement with the predicted target size of 91 bp from samples containing DNA isolated from the MRSA strain, Mu50, from 5×10^5 copies down to 5 copies (Fig. 3A, lanes 2 – 7, upper panel). The negative control sample did not contain a 100 bp band (Fig. 3A, lane 8, upper panel). A positive test line was also seen on the DNA test strip from the corresponding positive samples (Fig. 3A, lanes 2 – 7, lower panel) but not from the negative control sample (Fig. 3A, lane 8, lower panel). This demonstrates that the detection sensitivity of the lateral-flow asymmetric HDA assay is similar to what is obtained from agarose gel analysis with the limit of detection of 5 copies of MRSA Mu50 DNA.

Correct product was also observed on both agarose gel and lateral-flow strips from samples containing 10^6 MRSA cells (Fig. 3B, lane 10), 100 MRSA cell (Fig. 3B, lane 12), and sample containing

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100 MRSA cells plus 10^6 non-resistant SA cells (Fig. 3B, lane 13). This indicates that asymmetric HDA can selectively amplify MRSA cells in the presence of non-resistant SA. Meanwhile, although primer-dimers were generated from the sample only containing 10^6 non-resistant SA cells (Fig. 3B, lane 11, upper panel), no positive signal was observed on the DNA lateral-flow test strip (Fig. 3B, lane 11, lower panel). This example demonstrates that probe-based lateral flow assay coupled with asymmetric HDA distinguishes MRSA-specific amplicons from non-specific products formed such as primer-dimers.

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What is claimed is:

1. A method for detecting a target polynucleotide segment in a sample, comprising:

- 5 (a) obtaining a single-stranded amplicon using helicase-dependent amplification (HDA) and a first primer at a concentration of at least three times greater than the concentration of a second primer wherein the first and second primers hybridize to opposite strands at opposite ends of the target polynucleotide segment; and
- 10 (b) hybridizing a single-strand probe to the single-stranded amplicon so as to detect the target polynucleotide segment.

2. A method according to claim 1, wherein the amplified target polynucleotide segment is diagnostic for a pathogen or a mutation

15 in a genomic DNA.

3. A method according to claim 2, wherein the genomic DNA is human genomic DNA.

20 4. A method according to claim 1, wherein the amplification is real-time amplification and the probe is a labeled probe.

5. A method according to claim 4 wherein the labeled probe is a fluorescent probe.

25 6. A method according to claim 1, wherein the amplification is detected by end-point analysis using either unlabeled or labeled probes.

30 7. A method according to claim 6 wherein end-point analysis is achieved by a lateral-flow device.

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8. A method according to claim 1, wherein the HDA is isothermal and performed between 20°C and 75°C.

5 9. A method according to claim 8, wherein the HDA is performed within a range of 60°C and 66°C.

10. A method according to claim 1, wherein the target polynucleotide segment contains a single nucleotide polymorphism.

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11. A method according to claim 1, wherein the second primer is provided at concentration within a range of 25 nM -75nM.

12. A method according to claim 1, wherein the helicase in the HDA is Tte UvrD helicase.

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13. A composition, comprising: a first primer and a second primer, wherein the ratio of the first primer is at least 3:1 with respect to the second primer, the first and second primer being capable of hybridizing to opposite strands of a polynucleotide sequence at opposite ends of a target polynucleotide segment; and a helicase in an effective concentration for amplifying a polynucleotide to produce a single-strand amplicon.

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14. A composition according to claim 13, wherein the second primer has a concentration in the range of 25 nM -75nM.

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15. A composition according to claim 13, wherein the ratio of the first primer to the second primer is 4:1.

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16. A composition according to claim 13, wherein the helicase is Tte UVrD helicase.

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FIG. 1B

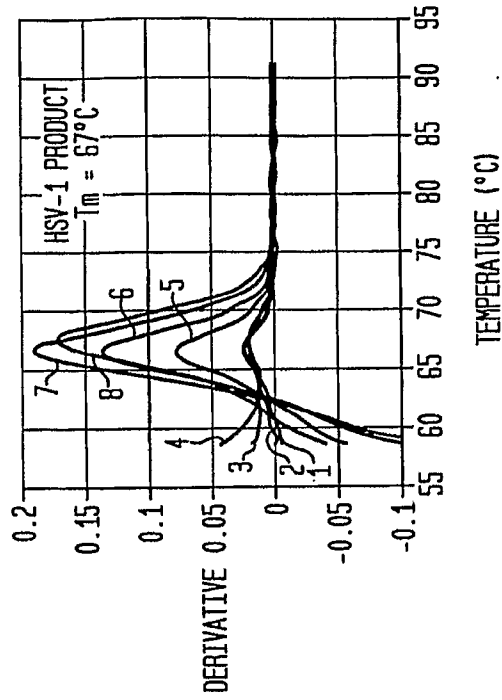
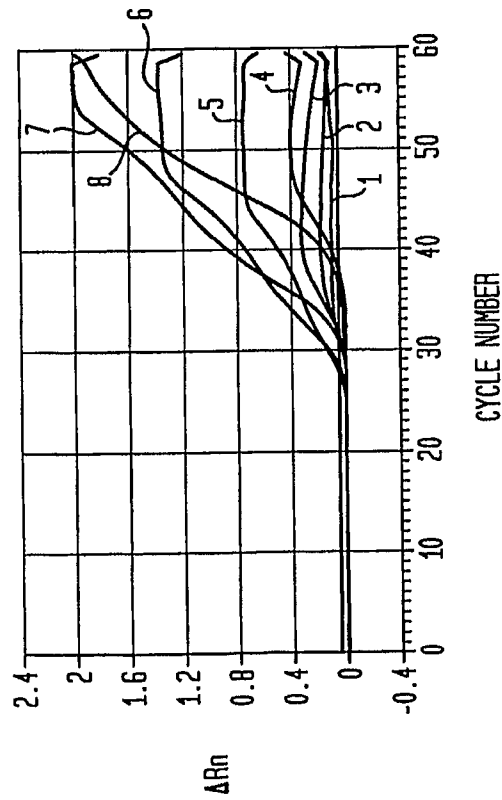


FIG. 1A



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FIG. 2A

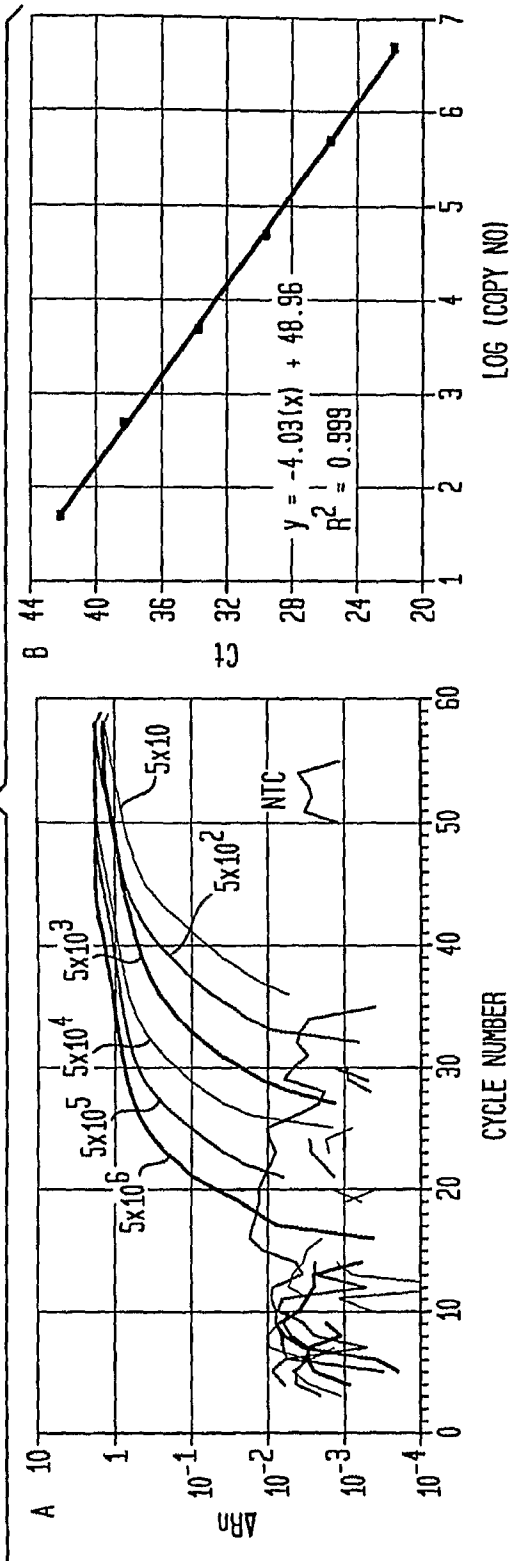


FIG. 2B

